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Research Article

Amplification of Tumor Transcripts from Limited Quantity of Esophageal Squamous Cell Carcinoma Tissue Samples

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Abstract

To examine the template-switching technology accompanied by in vitro transcription (the Switch Mechanism At the 5′ end of Reverse Transcript) to amplify enough amount of mRNA as input for gene expression experiments. We amplified limited quantity of esophageal squamous cell carcinoma (ESCC) transcripts samples using generated ds cDNA as template and in vitro transcription (IVT) reaction. In addition, the quality and quantity of amplified mRNA were assessed by comparative real-time PCR of genes such as stem cell markers CD44, OCT4 and SNAIL as well as MAGE-A4 as a cancer-testis antigens, and XRCC5 as an underexpressed gene in ESCC.

The results obtained from this study demonstrated that optimal amounts of mRNA are generated by template-switching and IVT reaction. Integrity and purity of all RNA samples were assessed. By using this approach, over 10 micrograms of amplified mRNA were generated from 100 ng of starting total RNA. The results of comparative real-time PCR of five genes with different levels of expression illustrated that the expression level of amplified sense RNA was almost similar when compared with non-amplified RNA. Our results clearly showed the usefulness of the T7-based IVT technique for amplification of limited quantity of input total RNA.

Keywords: mRNA amplification; in vitro transcription; SMART; ESCC

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Introduction

Identifying differentially expressed genes have emerged as a vital point for understanding the complex molecular mechanisms including cell growth, progression, differentiation and tumorigenesis and gene regulatory pathways. Evaluation of transcriptome profiling is a valuable tool to identify novel and key biomarkers involved in the biological procedure. There are numerous technologies available for characterization of differential gene expression. These technologies include microarray, RT-PCR, northern blot, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MAPS), differential display RT-PCR, and subtractive hybridization[1, 2]. Investigation of the gene profiling, as well as recognizing key markers in diagnosis, prognosis, progression and treatment of diseases, and all processes that lead to disease development require considerable amount of input RNA for transcript profiling analysis[3, 4]. Unfortunately, many of the specimens obtained from procedures such as laser capture microdissection, cell sorting, or biopsy samples are limited to a small-scale of tissue; for this reason, RNA obtained from clinical samples are a finite amount with low yield[5]. Several techniques are available for RNA amplification, but it is remarkable that in all these methods, no changes in the expression levels of genes should occur following amplification. Enrichment and amplification of limited amount of RNA should not change primary function of the RNA[6]. Most of mRNA amplification techniques are based on in vitro transcription (IVT) mechanism using the minimal quantities of RNA as starting sample[7]. The combination of template-switching (Switch Mechanism At the 5′ end of Reverse Transcript) technology with T7 RNA polymerase transcription is applied as one of the methods of RNA amplification. In this RNA amplification procedure, microgram amounts of mRNA have been obtained from nanogram or picogram quantities of total RNA. To amplify the extra amounts of mRNA, the RNA obtained from the IVT reaction is generated using second strand cDNA (ds cDNA) as template. Primary step for amplifying the transcriptome, the full-length single strand cDNA (ss cDNA), is generated through two oligonucleotide primers. First, a modified oligo (dT) primer (CDSIIA) is applied for ss cDNA synthesis, which is complementary to the SMART T7 oligonucleotide primer. Afterward, SMART T7 anchored to ss cDNA plus RNase H and DNA polymerase is applied to produce the ds cDNA with high quality. Eventually, linear sense RNA is amplified using T7 RNA polymerase and the ds cDNA template[8-10]. It is important to recognize that in the SMART amplification, what can change the expression level of genes after amplification of sample RNA. Because it is essential in further analysis, relative expression levels of individual genes after amplification should be similar to the original RNA level [11].

In the present study, we assessed that weather in vitro transcription reaction can generate sufficient amplified sense RNA from the finite amounts of total RNA via the application of the SMART methodology. Meanwhile, we comparatively studied the gene expression of known genes before and after RNA amplification of specimens. By using real-time RT-PCR as a comparative gene expression technique, the expression levels of mRNA in human esophageal squamous cell carcinoma (ESCC) tissue samples can be investigated.

Materials and Methods

Study population and sample collection

The human fresh tumoral and distant tumor-free tissues were collected from five patients with ESCC as RNA source through esophagectomies at Omid Oncology Hospital of Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. None of the patients received therapeutic procedure such as chemo- or

radiotherapy prior to surgery. The specimens were immediately transferred to RNAlater solution (Qiagen, Hilden, Germany) after esophagectomy and stored in -20^0 C. All samples histologically contained at least 70% tumor cells. The study was approved by the Ethics Committee of the MUMS.

Total RNA preparation

Total RNA was isolated from the five tissue samples by RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of extracted total RNAs was evaluated using the NanoDrop spectrophotometer based on the recommendations of the manufacturer, and also the absorbance ratios (A260/280) of 1.8 to 2.0. Additionally, the integrity of RNA was confirmed by illustration of perfect 28S and 18S ribosomal bands following electrophoresis on agarose gel.

SMART method

SMART –step 1. To synthesize first-strand cDNA, a mixture reaction containing 200 ng of total RNA prepared from normal or tumoral tissues of ESCC samples as a template that was combined with 1 µl of 12 µM cDNA synthesis (CDS) primer II A and deionized water for a final volume of 4.75 µl. Specific oligonucleotide sequences are listed in Table 1. The solution was heated at 70^0 C for 5 min and then snap-cooled on ice. Then the reaction was followed by the addition of 2 µl 5X first-strand buffer (Thermo Scientifc, USA), 0.5 µl of 100 mM dithiothreitol (DTT), 0.25 µl of 40 U/µl Ribolock RNase inhibitor (Thermo Scientifc, USA), 1 µl of 10 µM SMART T7 oligonucleotide, 1 µl of 10 mM dNTP (Pars tous, Iran) and 1 µl of 2000 U/µl Maxima H Minus Reverse Transcriptase (Thermo Scientifc, USA) to the RNA solution. Afterward, temperature was adjusted at 37˚C for 2 hours to start the first-strand cDNA synthesis, followed by terminating the reaction at 68˚C for 10 min.

SMART –step 2. For the synthesis of double-strand cDNA, the second strand was synthesized in a total volume of 100 µl reaction mixture including 73 µl deionized water, 10 µl of 10X PCR buffer (Pars tous, Iran), 2 µl of 10 mM 50 X dNTPs mix, 2 µl of 10 µM T7 extension primer (Table 1), 2 µl of 5 U/µl RNase H (BioLab, England) and 2 µl Taq DNA polymerase (Pars tous, Iran) were added to the first-strand cDNA sample. The solution was incubated at 37˚C for 15 min, 95˚C for 5 min, 59 ˚C for 1 min and 72˚C for 15 min.

Table 1 Oligonucleotides sequences used for ss cDNA and ds cDNA synthesis

Oligonucleotides	Sequence (5'→3')
cDNA synthesis (CDS) primer II A	AAGCAGTGGTATCAACGCAGAGTACT(30) VN
SMART T7 Oligonucleotide	ACTCTAATACGACTCACTATAGGGAGAGGGCGGG
T7 Extension Primer	GCTCTAATACGACTCACTATAGG

(N = A, C, G, or T; V = A, G, or C)

SMART –step 3. To purify double-stranded cDNA, the obtained ds cDNA was purified by PCR cleanup kit (Qiagen, Hilden, Germany) according to the user manual. Finally, the sample eluted in 12 µl deionized water. Then 2.5 volume of (30 µl) 100% ethanol, 0.1 volume of (1.2 µl) sodium acetate (3 M) and 2 µl glycogen were added and the sample placed in a -20˚C freezer for overnight to precipitate the cDNA. The obtained cDNA pellets were rinsed with 70% ethanol, dried, and then precipitated cDNA was resuspended in 10 µl RNase-free water.

SMART –step 4. To synthesize mRNA by T7 in vitro transcription, the T7 transcription kit (Jena Bioscience, Germany) was used. In brief, the purified double-strand cDNA solutions were added to a mixture reaction

including 4 µl of T7 reaction buffer, 1 µl of NTP mix, 0.2 µl of 40 U/µl RNase inhibitor and 0.2 µl of 200 U/µl T7 RNA polymerase in a 20 µl reaction volume that filled up with water. The solution was incubated at 37˚C for 12 hr.

SMART –step 5. The synthesized mRNA was purified using 2.5 volume of 100% ethanol, 0.1 volumes of sodium acetate (3 M) and 2 µl glycogen, and the sample placed in a -20˚C freezer for overnight to precipitate the mRNA. The obtained pellets were washed with 70% ethanol and reaction pellet was diluted in 10 µl RNase-free water.

Electrophoresis of mRNA synthesized by the SMART method

To confirm the synthesis of mRNA, electrophoresis was performed on 1.2% agarose gel at 100 V for 30 min. Also, the concentration of the mRNA was determined using the NanoDrop spectrophotometer.

RT-PCR

The quality of amplified mRNA was determined by using three housekeeping genes including Glyceraldehyd 3-phosphat dehydrogenase (GAPDH), β actin and β2 *microglobulin* as positive control.

Real-time PCR

Amplification and relative expression ratios of synthesized RNA were confirmed by gene expression of the primer pairs using real-time RT-PCR. The primers were designed by AlleleID software 6.0 (PREMIER Biosoft, CA, USA). Specific primer sequence of the selected genes and PCR product sizes are listed in Table 2. Approximately 500 ng of RNA was used for the reverse transcription-polymerase chain reaction (RT-PCR) to obtain first-strand cDNA derived from RNA with oligodT primer using first-strand synthesis kit (Fermentas, Lithuania) in 20 µl reactions according to the manufacture's protocol. Each experiment was carried out in triplicate and repeated twice for each gene by using SYBR-Green method (Ampliqune, Denmark) in a Stratagene Mx3000P thermocycler (Stratagene, La Jolla, CA). Expression of GAPDH as an endogenous control was used to normalize the data. Based on previous studies, we selected several genes including MAGE-A4 (Melanoma-associated antigen 4), OCT4 (octamer-binding transcription factor 4), SNAIL (snail family zinc finger 1), XRCC5 (X-ray repair complementing defective repair in Chinese hamster cells 5), and CD44, that had shown different expression levels in the esophageal tissue [2, 12-15].

The following thermal cycling program for OCT4 and SNAIL were 10 min at 95˚C, followed by 40 cycles each of 95˚C for 30 sec, 59˚C for 30 sec, and 72˚C for 30 sec. The thermal profile for CD44S included 10 min at 95 ℃ as initial denaturation step, followed by 40 cycles of 15 sec at 95 ℃, 60 sec at 60 ℃, and finally 45 sec at 72 ℃. Thermal profile for MAGE-A4gene was applied as initial denaturation step 10 min at 95 ℃, followed by 40 cycles of 15 sec in 95 ℃, 30 sec at 60 ℃ and finally 72 ℃ for 45 sec. The subsequent thermal cycling program for XRCC5 gene was 3 min at 50 ℃, then 10 min at 95 ℃ as initial denaturation step, followed by 40 cycles of 15 sec at 95 ℃, 10 sec at 60 ℃, and 72 ℃ for 20 sec. The mRNA expression was compared in the non- amplified sample and same amplified sample.

Table 2 The genes and primer set sequences used for real-time PCR

Primer	primer sequence (5'→3')	Size of PCR products (bp)
GAPDH	F: GGAAGGTGAAGGTCGGAGTCA	101

	R: GTCATTGATGGCAACAATATCCACT	
SNAIL	F: ATGCCGCGCTCTTTCC R: TCAGCGGGGACATCCTG	118
OCT4	F: CCTGAAGCAGAAGAGGATCA R: CCGCAGCTTACACATGTTCT	148
CD44	F: TCCAACACCTCCCAGTATGACA R: GGCAGGTCTGTGACTGATGTACA	83
XRCC5	F: GCTTTTCCTCATATCAAGCATAACT R: AATCTCTGAAATCGAGGATTTGG	247
βactin	F: AGAGCTACGAGCTGCCTGAC R: AGCACTGTGTTGGCGTACAG	184
β 2 microglobulin	F: GGCACAACAGGTAGTAGGCG R: GCCACAGGAGCTTCTGACAC	170

Results

Assessment of quality of obtained RNAs

Briefly, total RNA was extracted from the tissue samples of patients with esophageal cancer, and then using electrophoresis and the measurement of absorbance, the quality of total RNA was estimated. The 260/280 nm absorbance ratio was generally in the range of 1.8 to 2 for total RNA that indicates the purity of the RNA. Moreover, RNA concentration was calculated using the Beer-Lambert law and the absorbance of RNA sample at 260 nm. In addition, by electrophoresis on 1.2% agarose gel the bands of 18S and 28S ribosomal RNA was confirmed for investigating the integrity of total RNA. Both integrity and purity of total RNA were appropriate for performing IVT reaction. Moreover, amplified mRNA was detected by electrophoresis on 1.2% agarose gel. In this experiment, we used 100 ng of starting total RNA sample in SMART amplification for 12 hours to obtain additional quantity of mRNA. The results displayed that the size of sense amplified RNA ranged between ~ 0.2 to 3.0 kb (data not shown). In addition, the ratios of absorbance for mRNA obtained at 260/280 nm were ~ 2.0 that is accepted for purity of generated mRNA.

Correlation between the quantity of starting samples and RNA obtained from T7 in vitro transcription

The aim of this research was to examine the mRNA amplification method for generating large amount of mRNA. The method utilized was based on template-switching. Accordingly, tight controls of all steps were required to determine the applicability of T7-based SMART amplification procedure.

There is correlation between the starting amounts of RNA for in vitro transcription (IVT) and amplified RNA. Reaction of IVT was carried out using 100, 1000 and 5000 ng of total RNA as the initiating specimen. As shown in Table 3, IVT reaction was performed for 12 hour. The mRNA was amplified from 100 ng of total RNA when IVT reaction was performed for 12h. The yield of RNA obtained from IVT depends on the amount and purity of starting RNA template. The results showed that there is a direct association between the

amount of primary and obtained RNA.

Table 3 Quantity of mRNA amplified in IVT reaction

Quantity of starting samples (ng)	Quantity of obtained mRNA by in vitro transcription (12 h)
100	12000 ng
1000	18000 ng
5000	22000 ng

Detection of synthesized mRNA

First, the target amplified mRNA was assessed by RT-PCR with the expression of three housekeeping genes. After total RNA extraction and mRNA amplification, 500 ng samples were reverse transcribed for cDNA synthesis to evaluate the presence of GAPDH, β actin and β2 *microglobulin genes* by RT-PCR. The presence of these genes was confirmed by RT-PCR in both samples through the size of amplicons that were illustrated on agarose gels.

As the second step for confirming the change in gene expression by the SMART method, the comparative real-time PCR of CD44, MAGE-A4, SNAIL, OCT4 and XRCC5 genes was performed using RNA obtained before and after amplification. The expression levels in amplified and non-amplified RNA were compared together as shown in Figure 1. The results of relative expression ratios of the examined genes, before and after amplification, were similar.

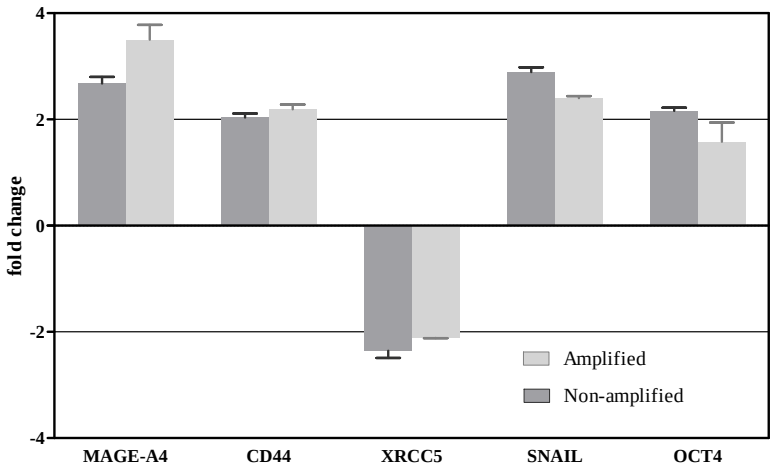

Figure1 Examination of change in the comparative gene expression by the SMART mRNA amplification in shown.

Discussion

Various clinical researches have required plenty amounts of RNA from limited amount of biological samples to perform analysis of gene expression. Therefore, it is crucial that the fidelity of amplified RNA in great demand is preserved to the expression profiles of total primary RNA. There are numerous methods that can amplify the scant amount of total RNA or mRNA for subsequent important applications. The signal and

global mRNA amplification are two principal ways for RNA amplification. Both of in vitro transcription and PCR amplification were introduced as two different procedures of global mRNA amplification, which differ based on basic mechanisms of generation of ds cDNA as a template. Generation of ds cDNA via a switch-template mechanism at 5' end of the transcript can reserve the original amplified RNA versus total RNA. The production of ds cDNA is introduced as an essential step in RNA amplification**.** Sense and antisense orientation in which the obtained RNA can be employed in gene expression approaches are among different methods of linear RNA amplification. Both of these methods can increase the number of transcripts from an insignificant starting quantity. Applications of antisense RNA include gene expression profiling, cDNA microarray and cDNA library construction as well as downstream proteomic applications. However every method has some obstacles, as antisense RNA cannot be used for hybridization with reverse transcription labeling approaches. The sense RNA can be applied for microarray analysis of gene expression. cDNA synthesis from amplified sense RNA can be applied as a hybridization target for any analysis with a sense-strand trend such as oligo microarray[4, 8, 16-18]. The present study has evaluated the technique of amplified sense RNA, in order to generate the high quantity of mRNA from finite amounts of tissue RNA available as starting sample. Here, sense RNA amplification was performed based on the switch-template and linear T7 in vitro transcription. In vitro transcription is an approach that synthesizes mRNA sequences with any quantities, and it has been used in techniques such as hybridization analysis, analysis of RNA-proteins binding, or as functional molecules[19]. IVT by using ds cDNA as a source template for amplification is introduced as an efficient approach for amplification of mRNA sequences[10]. We can utilize both total RNA and mRNA as starting sample for IVT reaction.

The quantity and quality of the starting RNA are variable and strongly rely on separation and purification techniques of RNA. High quality RNA isolation is among the critical steps for downstream genetic analysis[20, 21]. We used the quantity of less than 100 ng of total RNA with high quality, and the results demonstrated substantial amounts of amplified mRNA. In addition, the findings illustrated that the most amounts of mRNAs were obtained when the IVT reaction was fulfilled for 12 h. In this study, fresh tissue samples of ESCC patients were used as input source for RNA isolation and amplification. The results indicated that the amount and quality of the starting sample and also the reaction time of IVT can affect the quantity of generated mRNA. There is a close correlation between the initial concentration of used RNA and quantity of RNA obtained from amplification procedure[22].

IVT fidelity can be determined through the comparative gene expression ratio, which is defined as unamplified in comparison to amplified RNA [23]. Here we compared the efficiency of generated mRNA via comparative real-time PCR versus total RNA. The expression level of described genes including MAGE-A4, CD44, XRCC5, SNAIL and OCT4, were obtained by real-time PCR using generated mRNA from IVT. In previous studies, expression level of these genes had also reported[2, 12-15]. Our results of real-time PCR were revealed that expression level of genes in amplified RNA was almost same with unamplified RNA. As a whole, our data have supported that the expression level of genes in transcripts generated by the SMART amplification approach are very similar.

In conclusion, we demonstrated that mRNA amplification using the T7-based linear amplification method as a simple and rapid procedure was performed to generate considerable amount of RNA from a limited quantity of starting sample. The results of present study also showed similarity between the expression ratios of the examined genes in the amplified sample versus unamplified. Due to the low percentage of tumor tissue stem cells, this approach can be successfully applied for amplification of genes in cancer stem cells without change in their expression level.

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